



PCT/GB 2003 / 003558



## PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

The Patent Office

Concept House

Cardiff Road

Newport

South Wales

NP10 8QQ

REC'D 30 SEP 2003

WIPO

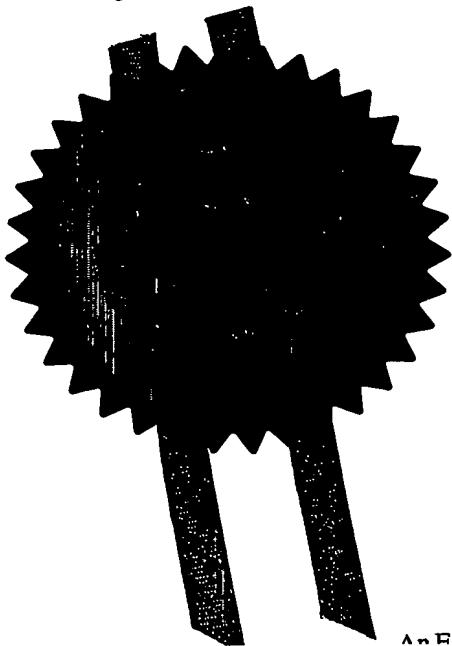
PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Dated

*R. Whalley*  
22 September 2003

Patents Form 172

Patents Act 1977

(Rule 16)



**Request for grant of a patent**

23AUG02 E743129-2 D02611  
P01/7700 0.00-0219618.6

The Patent Office  
Cardiff Road  
Newport  
South Wales NP10 8QQ

1. Your reference  
**5440401/JAC**

2. Patent Application Number

**22 AUG 2002** **0219618.6**

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

**Battelle Memorial Institute**  
505 King Avenue  
Columbus  
Ohio  
43201-2693  
USA

Patents ADP number (*if known*)

**0599438 9001**

If the applicant is a corporate body, give the  
country/state of its incorporation

Country: United States of America  
State: Ohio

4. Title of the invention

**WOUNDCARE**

5. Name of agent

**Beresford & Co**

"Address for Service" in the United Kingdom  
to which all correspondence should be sent

**2/5 Warwick Court**  
**High Holborn**  
**London WC1R 5DH**

Patents ADP number

**00001826001**

6. Priority details

Country

Priority application number

Date of filing

**Patents Form 1/77**

---

7. If this application is divided or otherwise derived from an earlier UK application give details

Number of earlier application

Date of filing

---

8. Is a statement of inventorship and/or right to grant of a patent required in support of this request?

Yes

---

9. Enter the number of sheets for any of the following items you are filing with this form.

Continuation sheets of this form	0
Description	16
Claim(s)	0
Abstract	0
Drawing(s)	3 + 3 S

---

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent ( <i>Patents form 7/77</i> )	1 + 1 copy
Request for preliminary examination and search ( <i>Patents Form 9/77</i> )	0
Request for Substantive Examination ( <i>Patents Form 10/77</i> )	0
Any other documents (please specify)	0

---

11. I/We request the grant of a patent on the basis of this application

Signature Beresford in  
BERESFORD & Co

Date 22 August 2002

---

12. Name and daytime telephone number of  
person to contact in the United Kingdom

Jane Anne Clark

Tel: 020 7831 2290

## WOUNDCARE

This invention provides methods to use porous biocompatible, biodegradable and/or bioresorbable fibrous polymeric materials, such as scaffolds/matrix, generated by an electric field effect technology (EFET), for facilitating cell attachment, movement, growth, and differentiation, and formation of tissues such as bone, cartilage and tendon.

The resultant electric field used in the EFET process is controlled and used to direct the fibres to a target surface, such as an area of skin damage so as to lay down a patterned mat of fibres, to form a scaffold or matrix. The scaffold is characterised by a three-dimensional continuous network of intercommunicating fibres, with controllable fibre diameter and gap size between fibres.

The type of pattern of the scaffolds may be varied, for instance, when the fibres are generated by EFET, using polymers formulated with solvents, the diameter of fibres ranges from 0.2 to 100 microns, with a gap size of about 10 to 500 microns. When the fibres are generated by EFET with molten polymers, the fibre diameter varies from 2 to 500 microns, with a gap size of about 25 to 3000 microns.

When fibres are ejected from the nozzle, the charged fibres move axially towards the target, say a damaged area, and arrive perpendicular to the surface. Immediately after the fibre touches a surface, the remaining fibre experiences a lateral force, due to repulsion of the layer that has settled but has not lost its electrical charge. The degree of the lateral force will be proportional to the amount of charge on the settled (non-moving) part of the fibre being laid down, and this is inversely proportional to the relaxation time of the polymer. In other words, if the polymer's relaxation time is brief, say a microsecond, a small lateral force will be developed on the moving fibre; and *vice-versa*. Lay down patterns have been observed to vary from roughly rectilinear, straight-line patterns, to a series of loops and circles.

The controlling parameters are: the nozzle voltage, the viscosity and resistivity of the polymer, in solution and upon spray drying, the evaporation rate of the solution (if formulated polymer solutions are used), and the mass flow rate of the fibre-jet. Reduced resistivity and/or high nozzle voltages lead to increased charge on the spun fibre. The dielectric constant and resistivity of the spun polymer could control the relaxation time, and suitable polymers may be chosen to give optimal relaxation periods.

The fibre scaffold thickness is limited by the repulsive forces that it exerts upon the moving fibre, as it attempts to settle on to the scaffold. Therefore, optimal settling and lateral forces may be adjusted accordingly. For example, if the relaxation time is optimised, say to a few milliseconds, then it will exert useful lateral forces in order to move the settling fibres, but will quickly allow the fibres to return and settle, so as to form a multi-layer scaffold of fibres.

Fibres may also have their charge raised or lowered by gas-ion bombardment. When bombarded with like polarity ions, either on the surface of the settled scaffold, or on the surface of the moving-settling fibre, the lateral forces may be increased, and *vice versa*.

The optimal gap size of the fibre scaffolds is cell type dependent, and may be important for cell movement, differentiation and growth, neo-vascularisation and production of extracellular matrix. For example, a fibre diameter between 1-10 microns could be optimal for growing skin fibroblasts and forming skin tissue, and for progenitor stem cells differentiating into another cell type without the addition of extrinsic proteins such as growth factors, and for cell proliferating in preferred growth rates.

It is believed that if the fibre diameter is of comparable, smaller dimension to the cell, a signal to grow in the preferred direction is established. This diameter, together with the polymer's surface chemistry and topography, are also believed to affect the signal that may accelerate or decelerate the growth rate and cell differentiation.

The pattern of the fibre scaffolds may act to signal cells to migrate to and move along the fibres. The lattice formation of fibres may be thus used to make fibroblasts form a weave pattern, rather than the aligned, parallel pattern that produces scar tissue. The fibroblasts will at the same time lay down a collagen basal layer for the next layer of skin to start the full process of tissue repair, therefore reducing the scar formation. Scar formation is believed to be due to an evolutionary action of cytokines that forms new skin as quickly as possible, to prevent infection. It can be shown that parallel, rather than interwoven patterns will do this.

The scaffolds have a lattice of internal space through which cell culture medium, bioactive factors, nutrients and gas can be supplied to the internal parts, and by-products can diffuse out, and therefore are conducive to cell attachment and maintenance of cell function. The scaffolds may also be used as body implantable materials, for supporting tissue growth, such as skin, bone, cartilage and tendon, *in vitro*, and *in situ* i.e at the injury sites. The bio-artificial tissues generated *in vitro* could be used for transplantation in wounds, dermal burns, bone fractures or cartilage degeneration.

In one aspect, the present invention provides a method of forming a wound dressing, which comprises using EFET techniques to produce electrically charged polymer fibres, which deposit on to the wound to form a dressing. This method comprises controlling the formation of the polymer fibres so as to control the polymer charge relaxation time and thereby the pattern in which the polymer fibres are laid down on the wound and/or skin, bone, cartilage.

In one aspect, the present invention provides a method of forming a wound dressing which comprises producing a polymer fibre or fibres using EFET techniques so that the polymer fibres deposit onto the surface of a target area, such as skin, and/or wound, to form a covering or dressing for the target area. This method comprises controlling the polymer fibre production so as to control the polymer charge and relaxation time, and thereby control the lateral force experienced by the polymer fibre resulting from the fibre that has already settled on the target area, so as to control the lay down pattern of the polymer fibre or fibres on the target area, to produce a lattice or web like polymer fibres to facilitate the formation of skin tissue by fibroblasts of a weave pattern rather than an aligned parallel pattern.

One feature of the invention is to use scaffolds generated by spraying polymer solutions formulated with solvents and/or by spraying molten polymer, for cell culture so that bio-artificial tissues such as skin, bone, cartilage, muscle and tendon can be formed. Examples of cells that could be used for cell culture are skin fibroblasts, osteogenic cells, progenitor cells, muscle cells and bone marrow stem cells. There is a vast potential for tissue regeneration from cells with stem cell characteristics. The development of osteoblasts, chondroblasts, adipoblasts, myoblasts and fibroblasts results from colonies derived from such single cells. They may, therefore, be useful for regeneration of all tissues that this variety of cells comprises: bone, cartilage, fat, muscle, tendons and ligaments.

Current methods using the EFET process involve spraying polymers that are formulated with one or more solvents, in such a way that droplets or fibres are formed when the solvents are evaporated in ambient air. To formulate certain type of polymers such as poly(3-hydroxybutyric acid) (Biopol), can only be formulated with toxic solvents such as methylene chloride, therefore the use of molten polymers for EFET spraying is important.

One feature of the invention is to use macroporous fibrous scaffolds generated by spraying molten polymer for culturing cells, such as osteogenic or progenitor cells in order to create bone, cartilage and tendon tissues. Scaffolds of fibre diameter such as 25 microns and gap size, for example, 150-200 microns may be suitable for stem cell and/or differentiated cell attachment, movement, differentiation, proliferation and formation of extracellular matrix. The network of these scaffolds aims to resemble the three-dimensional structure such as bone, cartilage and tendon.

One feature of the invention is to enhance regeneration of tissues by combining the principal of gene therapy with tissue engineering. This could be achieved by spraying a plasmid DNA carrying the gene for a protein or growth factor on to the fibre scaffolds, or by spraying polymer solution containing plasmid DNA so that the plasmid DNA is physically entrapped within the fibre scaffolds. The plasmid DNA may also carry a promoter/repressor gene so that the expression of the gene for the protein/growth factor can be turned on or off as desired. Fibre scaffolds containing plasmid DNA may enhance cell attachment and proliferation, and regeneration of tissues.

One feature of the invention is to spray mammalian cells /platelets, using the EFET process, for the delivery of live cells to wounded or defective tissues such as skin, bone, cartilage, tendon and cornea.

One feature of the invention is to spray biological micro-organisms, healthy cells, cultured cells or genetically engineering cells that express a therapeutic protein, directly onto a target area, such as skin, bone, cartilage, wounds and burns, for cell or gene therapy; or encapsulated into polymer fibres for tissue engineering such as bio-artificial skin, bone and cartilage, for controlled released delivery of proteins, enzymes, etc., to a target area, for enzyme or hormone therapy. When cells are encapsulated into fibres, they might be protected from immunological processes, and thus survive and maintain an effective supply of proteins, and therefore may be useful in enzyme or hormone therapy. When blood vessel cells such as endothelial cells are delivered to a fibre scaffold or an injury site, they may promote neo-vascularisation

and thus enhance the healing process. When blood clot formation cells such as platelets are delivered to a bleeding area, further blood loss could be prevented.

From a tissue engineering point of view, cells could either be sprayed or seeded to migrate into the fibre scaffolds, where they undergo cell proliferation and differentiation. Alternatively, scaffolds could be sprayed or seeded with genetically engineered cells that carry a plasmid DNA with a promoter/repressor gene (so that the level of expression of a protein can be controlled), before implantation to an injury site. By including a fibre scaffold, the level and duration of transgene expression by implanted cells may be enhanced.

**Example 1****Aim:**

1. To determine if human skin fibroblasts use polymer fibres as substrates for cell attachment and growth
2. To determine the relation between fibre diameter and cell size for optimal cell growth.

**Materials:**

## Cells used:

1. human skin fibroblasts
2. Chinese Hamster Ovary cells (CHO)
3. SV40-transfected African Green monkey kidney cells
4. human epitheloid carcinoma of the cervix (HeLa)
5. a human histiocyte lymphoma cells (U937) – non adherent cells

This range of cells was used to help obtain a general, more informed opinion, and therefore avoid having misleading results.

## Polymers used:

1. Newskin,
2. Eudragit RL100,
3. polycaprolactone (PCL, mol. wt. 65,000),
4. polylactide (L:D isomer = 50:50),
5. polylactide (L:D isomer = 96:4)

All the polymer fibres were generated by the EFET, on 22 x 22 mm glass coverslips of 0.16-0.19 mm thickness. Fibres were 1-5 microns in diameter (see table 1 for spraying parameters). Glass coverslips without polymer fibres were used as controls.

	Newskin	Eudragit RL100	PCL65	Polylactide (50:50)	Polylactide (96:4)
Formulation	As supplied	25% in ethyl alcohol	18.33% in acetone	3.33% in acetone	1.39% in acetone
Nozzle to plate distance (cm)	14	20	16.5	19	15.5
Flow rate (ml/hr)	4	3	12	8	12
Voltage (kV)	-22	-20	-28.5	-15	-15
Diameter (micron)	1-2	1-2	5	1-2	1-2

**Table 1.** Spraying parameters to generate polymer fibres for cell culture

All the polymer fibre coated-coverslips were sterilised with beta-irradiation at AEA Technology, Oxford. The plain coverslips were sterilised in 70% ethanol and then flame-dried before use. The polymer fibre coated-coverslips were pre-wet in phosphate to decrease the surface tension, before the cells were seeded ( $3 \times 10^4$  per coverslip) on the polymer fibres.

Procedure:

*Chemical nature of fibre surfaces*

It is thought that adhesion of cells to a surface is largely dependent on the chemical structure of a surface. This experiment was therefore designed in such a way that polymer fibre-coated coverglass were prepared from five different polymers and these coverglasses were put in a 150mm-culture dish. Chinese Hamster Ovary (CHO) cells were seeded on top of the fibres and the dish. This was to determine the effect of different substrates on cell adhesion.

*Measurement of cell proliferation*

Proliferation or metabolism of human skin fibroblasts was measured using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. The MTT assay measures the amount of an enzyme succinate dehydrogenase, SDH (a stable cytosolic enzyme that is released upon cell lysis), which converts the tetrazolium salt into an insoluble purple-blue formazan product. The absorbance of each sample was then read at 570nm, and the intensity of the purple-blue colour that appears should be directly proportional to the number of viable cells.

*Cell morphology*

The morphology of the cells grown on the polymer fibres was examined under light and phase contrast microscopes.

Results

*Chemical nature of fibre surfaces*

Results showed that the plating density (the number of cells settled down per unit area) on all the surfaces appear to be similar, implying that cells do not have a preference for a surface chemical structure.

*Cell adhesion*

In the coverslips without polymer fibres, it was observed that the human skin fibroblasts adhered to the surface of the coverslips, and their processes spread completely. They tend to form close parallel arrays as they approach confluence.

All the adherent cells were found to attach to and align with the fibres of polymer 1, 2, 4 and 5. Also, cells did not migrate to the glass space in between these polymer fibres. Fibres prepared from polymer 5 appear to be the best substrate, as all cell types showed good growth along these fibres (figure 1). On the contrary, only a few cells were found adhered to fibres of polymer 3 and most of them grew on the glass space in between the fibres. This indicates that adherent cells preferred to use the fibres of polymer 1, 2, 4 and 5 as substrates.

The diameter of the fibres of polymer 3 (polycaprolactone) was the largest (5 microns) compared to the fibres made from the other polymers (1-2 microns). This indicates that the diameter of a fibre may be a key factor for cell adhesion and growth. Cells could be able to recognise fibres of a small diameter, such as 1-2 microns, as a curved surface and attach to them. This hypothesis is consistent with the findings that stronger growth of cells was found on a rough surface that was prepared by painting with polymer solutions than on coverglass surface.

The non-adherent human histiocyte lymphoma cells (U937) was used as a control cell line. Results showed that these cells continued proliferating and did not adhere to any of the fibres.

Results from a preliminary experiment showed that CHO cells adhered to those fibres hanging over the edges of the coverslips. These hanging fibres are especially prevalent with polymer 1 and 3. However, most of the cells crawled through the polymer 3 fibre mat on coverslips and grew preferentially on the surface of the coverslips.

It is known that adhesion proteins play an important role during cell adhesion, for example, L-selectins on the lymphocytes surface specifically bind to carbohydrates on the lining of lymph node vessels. Protein molecules present in human serum may therefore help binding of cells to the polymer fibres. To verify this idea, fibre-coated coverglasses were submersed in normal human serum and incubated at 37°C. Proteins were extracted from the fibres and analysed on a polyacrylamide gel electrophoresis system. In order to visualise any protein bands present, the gel was stained with a very sensitive dye – silver nitrate. Interestingly, it was found that a protein of about 20 kD bound to the polymer fibres. The identity of the proteins remains to be determined by protein sequencing.

#### *Cell proliferation*

The cells on all the fibre mats grew over a period of about 14 days and gradually became sub-confluent, indicating that cell proliferation had occurred. Proliferation of cells was confirmed by the MTT assay, with human skin fibroblasts. Results showed that the purple-blue colour increased over a period of seven days, indicating that the polymer mat provides a biological substrate to which cells can adhere and grow.

#### *Cell morphology*

All the adherent cells grew on the five types of fibre mats at a similar rate, and no signs of cell lysis and toxicity were identified. They also had normal morphological characteristics when examined using light and phase contrast microscopes.

#### Discussion

##### *Normal cell morphology*

In this study, we have demonstrated that human fibroblasts adhered and proliferated on polymer fibre mats prepared from polymers 1, 2, 4 and 5. The cells showed normal morphology, and no evidence of cytotoxicity was detected.

##### *Shape of fibres*

Apart from the chemical structure, it is increasingly being realised that the surface topography, especially on a fine scale, plays a vital role in the attachment of cells. Our results showed that cells preferred to elongate in the direction of a fibre. This finding was confirmed with the results using CHO cells on fibres of polymers 1 (Newskin) and 3 (polycaprolactone).

##### *Size of fibres*

Cell diameters are in the range of 2-20 microns; for example fibroblasts have a diameter of about 10 microns. Cells also have membranes with thickness of about 100nm and cells surface receptors (10-100nm) that control the interactions with their

substrates. It is therefore conceivable to imagine that cells would like to attach to surface features that are about the same size as that of a cell receptor. As the size of the fibres of polymers 1 (Newskin), 2 (Eudragit RL100), 4 (polylactide 50:50) and 5 (polylactide 96:4) is about 1-2 microns, it is not surprising to see that cells find ways to adhere to the fibres and grow along the fibres. Also, the fibres are of a small diameter so that cells would be able to recognise they are on a curved surface. This curved surface could be of similar shape to part of an adhesion molecule. It is possible that cells may also prefer fibre diameters of less than 1 micron, for example, 10-100nm.

Whether cells grow along the fibres depends on the size of cells, the size of fibres and the gap size of the fibre scaffolds. If cells are very large compared to the size of the fibres and the gap size of the scaffolds, cells may tend to adhere to the top of a few fibres with no cell migration occurring.

#### *Signal sent to cells*

How do cells know about the dimensions of the substrates? A cell membrane has a close interaction with the internal cytoskeleton. The cytoskeleton is composed of actin microfilaments, intermediate filaments and microtubules, which give shape to a cell, provide support for cell extensions, and are involved in cell movement and interactions with the substratum on which the cell is lying. Any change in the substrate, for example, a weave pattern of fibre scaffolds, will affect the generation of signals within the cell and cause some kind of activation process that results in the changing of cell shape.

#### Conclusions

Taken together, human skin fibroblasts preferred to adhere to and grow on polymer fibres of diameter of 1-2 microns. The diameter of a fibre appears to play a key role in cell adhesion and growth, and act as a physical means for cell signalling, as it may activate appropriate signals within cells and cause some kind of activation process that results in the changing of cell shape. This process could also be enhanced in the presence of some adhesion proteins.

**Example 2**

Aim: to characterise the importance of the dimensions of fibres and type of polymers for the growth of human bone marrow fibroblasts (HBMF, osteogenic stem cells, 25 microns in diameter).

**Procedure:**

Three samples: Polycaprolactone with molecular weight 65,000 (PCL-65), Eudragit E100 and polymethylmethacrylate (PMMA) were used. Fibres were prepared by spraying three polymer solutions that were formulated in solvents (see table 2). Fibres were collected onto aluminium foil, to form scaffolds of about 1mm thick. Scaffolds were then removed for cell culturing.

	PCL65	Eudragit E100	PMMA
Formulation	20% in acetone (w/v)	40% in ethyl alcohol (w/v)	25% in acetone (w/v)
Nozzle to plate distance (cm)	10	22	5
Flow rate (ml/hr)	10	20	6
Voltage (kV)	10	20	11
Diameter (micron)	3	7.5	10
Gap size (micron)	16	50-200	32

**Table 2.** Spraying parameters to generate polymer fibres for culturing of HBMF cells.

The fibre diameter and gap size of each scaffold were determined using light and electron microscopes. The biocompatibility of the scaffolds was tested by seeding and growing HBMF cells onto the scaffolds for 7 days.

**Results**

All the fibres had very strong electrostatic charge, and adhered to plastic and metal surfaces, particularly tissue culture containers. Charged polymer fibres may help cell signalling, cell attachment, cell growth and tissue formation.

When examined under light and scanning electron microscopes, there were three different sizes of fibres for PCL-65 scaffold. The diameters of the fine fibres were about 3 microns with a gap size of about 16 microns.

The fibres of Eudragit E100 appeared to be transparent, and very homogenous. The diameter of the fibres was about 7.5 microns, with a gap size of about 50 to 200 microns.

As with Eudragit E100, the fibres of PMMA scaffolds were very homogenous. The diameter of the fibres was about 10 microns, with a gap size of about 32 microns.

Fibre scaffolds were saturated with culture medium before osteogenic stem cells were seeded. Experimental results showed that the PCL-65 scaffold was about 90% saturated, and cells were able to seed on the scaffold and survive.

The Eudragit E100 scaffold was saturated completely in the medium. However, the scaffold was dissolved in the culture medium, resulting in a very acidic culture environment. The microstructure of the Eudragit E100 scaffold appeared to be very homogenous and thus may be appropriate for used as a substrate to support cell attachment and maybe cell movement (figure 2). It is possible that cross-linking the scaffolds after spraying can make them insoluble in cell culture medium, and thus suitable for cell culture.

The PMMA is not a biodegradable polymer and the scaffold remained dry after 7 days, rendering it unsuitable for cell culture.

HBMF cells were then seeded onto PCL-65 scaffolds and cultured for 7 days. Half of the scaffolds were stained with toluidine blue for visualisation. The other half of the scaffolds were fixed in 4% formaldehyde/PBS, embedded in an OTC compound and frozen to -30°C for cryostat sectioning (figure 3).

Conclusions:

Scaffolds generated from PCL-65 are biocompatible to HBMF cells, as the morphology of the cells remained normal and no sign of cytotoxicity was detected.

**Example 3**

Aim: to study the interaction of human bone marrow fibroblasts (HBMF, osteogenic stem cells) with EFET scaffold and determine the cell morphology.

Having proved PCL-65 is biocompatible to cells (see example 2), scaffolds from PCL-65 were used for cell culture, in order to obtain additional information. Two other polymers: polylactide (isomer L:D=96:4) and Eudragit RL100 were also used.

Procedure:

Fibres were prepared by spraying three polymer solutions that were formulated in solvents (see table 3). Fibres were collected onto aluminium foil, to form a thickness of about 0.5mm thick. The fibre scaffolds were removed from the foil for cell culturing.

	PCL65	Polylactide (isomer L:D=96:4)	Eudragit RL100
Formulation	20% in acetone (w/v)	40% in ethyl alcohol (w/v)	22.5% in acetone (w/v)
Nozzle to plate distance (cm)	14.5	14.5	15.5
Flow rate (ml/hr)	10	28	3
Voltage (kV)	27	23	20
Diameter (micron)	3	3	10

**Table 3.** Spraying parameters to generate polymer fibres for culturing of HBMF cells.

EFET scaffolds were washed in water and soaked in phosphate buffered solution (PBS) overnight and then with cell culture medium. HBMF cells were seeded onto the scaffolds. HBMF cells were genetically labelled with a green fluorescent protein (GFP), using a nuclei transfer technique. The cells were grown in antibiotic G418 for selection. When the GFP was expressed in cells, rendering the cells fluorescence and thus are easy to visualise under microscopic examination. Cells were grown for 21 days, and examined using light, fluorescence and scanning electron microscopes, and focused ion beam technique on day 4, 7, 14 and 21. Scaffolds without cells were used as controls.

Results:

Results showed that HBMF cells were attached to the EFET fibres, with cell processes stretching along the fibres. The morphology of the cells appeared to resemble nerve cells (figures 4 and 5). This might suggest cell differentiation was occurring, without the addition of extrinsic biological factors. The topography of the fibre scaffolds, such as fibre diameter, could have an effect on cell phenotype in which it signals the stem cells to differentiate into another cell type, and to proliferate in preferred growth rates.

Cell growth was found on PCL-65 scaffold, and cell confluence was obtained at day 21. Similar to the cell growth on PCL-65 scaffold, many cells survived on the Eudragit RL100 scaffold. Cell growth was also found on polylactide scaffold,

although only a few cells survived. Some dead and fragmented cells were also seen. This suggests that HBMF cells may not be compatible with polylactide (isomer L:D=96:4).

Taken together, fibres generated from PCL-65 and Eudragit RL100 can be used as a substrate to support cell attachment and maybe cell movement, and HBMF cells appeared to prefer the PCL-65 scaffold. HBMF cells did not form abundant extracellular matrix on the scaffolds, and cell confluence was obtained only after 21 days in culture medium, suggesting that the network of the scaffolds, which is dictated by the fibre diameter and gap size, may not be optimal for cell proliferation. Scaffolds with gap size of at least 100 microns would allow cell penetration into the inner part of the scaffolds.

**Example 4**Aim: spray cell culture media with EFET processProcedure:

Culture medium, DMEM, was formulated with water-soluble polymer, polyethylene oxide (PEO, molecular weight = 100,000). The percentage of polymer present in the culture media, spraying parameters and results are summarised in table 4.

Results:

The presence of water-soluble polymer such as PEO, PVP and PVA could make aqueous formulations sprayable with EFET process. Cell culture medium, DMEM, was sprayed as polydispersed droplets when <14 % (w/v) PEO was present in the medium. When the percentage of PEO was  $\geq 14\%$  in the medium, fibres of diameter of about 2 microns and droplets of 70-100 microns were formed. No other additive was required, including surfactants.

**Table 4.** Spraying cell culture medium, DMEM, with EFET process

Formulation	Nozzle to plate distance	Flow rate	Voltage	Comments
0.6g PEO in 10 ml DMEM	2 cm	0.5 ml/hr	+11 kV	Droplets were formed, but the single jet was not very stable
0.8g PEO in 10 ml DMEM	2 cm	0.5 ml/hr	+11 kV	Droplets were formed, with very stable 1-2 jets.
	2 cm	0.8 ml/hr	+11 kV	Droplets were formed, with very stable 1-2 jets.
	2 cm	1 ml/hr	+11 kV	Droplets were formed, with unstable multi-jets.
1g PEO in 10 ml DMEM	2 cm	0.8 ml/hr	+11 kV	Droplets were formed (some with fibrils), with very stable multi-jets.
	2 cm	1 ml/hr	+11 kV	Droplets were formed (some with fibrils), with stable multi-jets.
	2 cm	1.2 ml/hr	+11 kV	Droplets were formed (some with fibrils), with unstable multi-jets.
	2 cm	1.5 ml/hr	+11 kV	Droplets were formed (some with fibrils), with unstable multi-jets
1.2g PEO in 10 ml DMEM	2 cm	1 ml/hr	+11 kV	Droplets were formed (some with fibrils), with very stable multi-jets.
1.4g PEO in 10 ml DMEM	2 cm	1 ml/hr	+11 kV	<2 microns beaded fibres and some 70-100 microns droplets, with unstable multi-jets.

	3 cm	1 ml/hr	+22 kV	<2 microns beaded fibres and some 70-100 microns droplets, with stable multi-jets.
	3 cm	2 ml/hr	+22 kV	<2 microns beaded fibres and some 70-100 microns droplets, with stable multi-jets.
	4 cm	1 ml/hr	+22 kV	<2 microns beaded fibres and some 70-100 microns droplets, with stable multi-jets.
	4 cm	2 ml/hr	+22 kV	<2 microns beaded fibres and some 70-100 microns droplets, with stable multi-jets.
1.6g PEO in 10 ml DMEM	5 cm	2 ml/hr	+23 kV	<2 microns fibres and some 70-100 microns droplets, with very stable multi-jets.
	6 cm	2 ml/hr	+30 kV	<2 microns fibres and some 70-100 microns droplets, with very stable multi-jets.
	6 cm	4 ml/hr	+30 kV	About 2 microns fibres and some 70-100 microns droplets, with unstable multi-jets.
1.8g PEO in 10 ml DMEM	5 cm	2 ml/hr	+23 kV	<2 microns fibres and some 70-100 microns droplets, with very stable multi-jets.
	6 cm	4 ml/hr	+30 kV	About 2 microns fibres and some 70-100 microns droplets, with unstable multi-jets.
2g PEO in 10 ml DMEM	5 cm	2 ml/hr	+23 kV	<2 microns fibres and some 70-100 microns droplets, with very stable multi-jets.
	6 cm	4 ml/hr	+30 kV	About 2 microns fibres and some 70-100 microns droplets, with unstable multi-jets.

**Example 5****Aim:** spray cell culture medium with starch corn**Procedure:**

Culture medium, Dulbecco's modified eagle's medium (DMEM), was formulated with polyethylene oxide (PEO, molecular weight = 100,000), and starch corn was used to mimic biological materials or cells. The amount of starch corn and percentage of polymer present in the culture media, spraying parameters and results are summarised in table 5.

**Results:**

Starch corn of about 10 microns in diameter was seen, together with droplets or fibres of the formulations used, depending on the percentage of PEO present in the formulations.

**Table 5.** Spraying cell culture medium, DMEM, with EFET process

Formulation	Nozzle to plate distance	Flow rate	Voltage	Comments
0.1g starch corn in 5ml 12% PEO/medium	5.5 cm	2 ml/hr	+30 kV	Droplets and starch corn were seen, with very stable multi-jets
0.1g starch corn in 5ml 20% PEO/medium	5.5 cm	2 ml/hr	+30 kV	About 2 microns fibres and some 70-100 microns droplets were formed, with very stable multi-jets. Some starch corn was incorporated into the fibres.
	5.5 cm	2.5 ml/hr	+30 kV	About 2 microns fibres and some 70-100 microns droplets were formed, with stable multi-jets. Some starch corn was incorporated into the fibres.

**Example 6**Aim: Spraying molten polymersResults

Polymers, for example, polycaprolactone (PCL, 65,000) were melted and moulded as solid sticks of 1.2 cm diameter and 20 cm in length. The polymer stick was inserted into an inlet tube of a hot gas gun, in which one end of the stick was in direct contact with a heating element. The temperature of the heating element was constantly maintained, such as at 204°C, by combusting butane gas. The outlet of the gun was a metal nozzle, which was close to the heating element. To the other end of the polymer stick, a syringe pump is directly attached to it, so that the stick was pressed downwards by the pump, and the flow rate of the molten polymer at the nozzle could be adjusted accordingly. The hot gas gun was positioned in such a way that the nozzle is pointing vertically downwards. To generate an electric field, the end of the nozzle was connected to a high voltage generator, and an earthed plate was located under the nozzle. Fibres were formed when the molten polymer was allowed to cool and solidify in ambient air.

PCL of molecular weight 65,000 was sprayed using this system, in which a single electrically charged polymer jet was collected on the earthed plate, as a continuous web of fibres ranging in size from 20-70 microns, and with a gap size of 100-500 microns. The results showed that, at a fixed distance between a nozzle and an earthed surface, the fibre diameter was dependent on the flow rate of the molten polymers (in table 6).

Flow rate (ml/hr)	Voltage (kV)	Distance between nozzle and earthed plate (cm)	Fibre diameter (microns)
4	23	25	50
5	22	25	60
2.5	16.5	22	70
2.5	10	23	70*

\*Fibres were collected on to an earthed rotating metal rod, with a diameter of about 1 cm.

**Table 6.** Spraying parameters of PCL (65,000) using the melt spraying method.



Figure 1. Cell growth of human fibroblasts along PCL-65 fibres. Magnification x200.



Figure 2. Eudragit E100 fibre scaffold. The diameter of the fibres is about 7.5 microns, and the gap size is 50-200 microns. Magnification x100.



Figure 3. Cells grown on PCL-65 scaffold for 7 days, fixed in 4% formaldehyde/PBS, embedded and frozen to -30°C for sectioning. The diameter of the fibres is 3 microns and the gap size is about 16 microns. Magnification x1000.

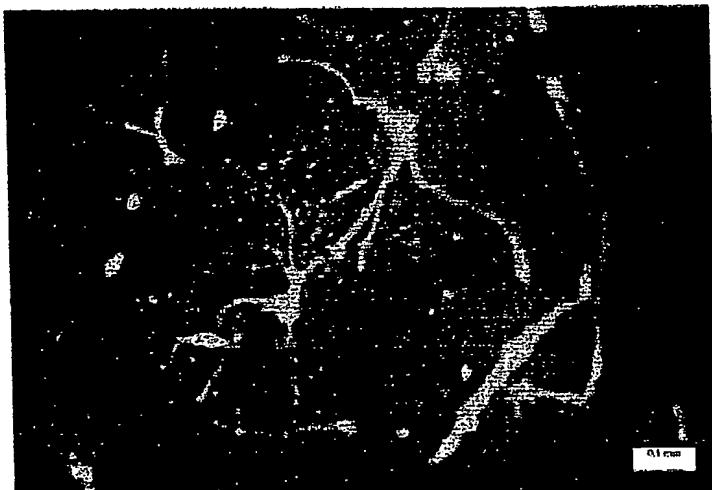


Figure 4. Cell growth of green fluorescent protein-labelled HBMF on PCL-65 scaffold after 7 days in cell culture medium. Magnification x100.

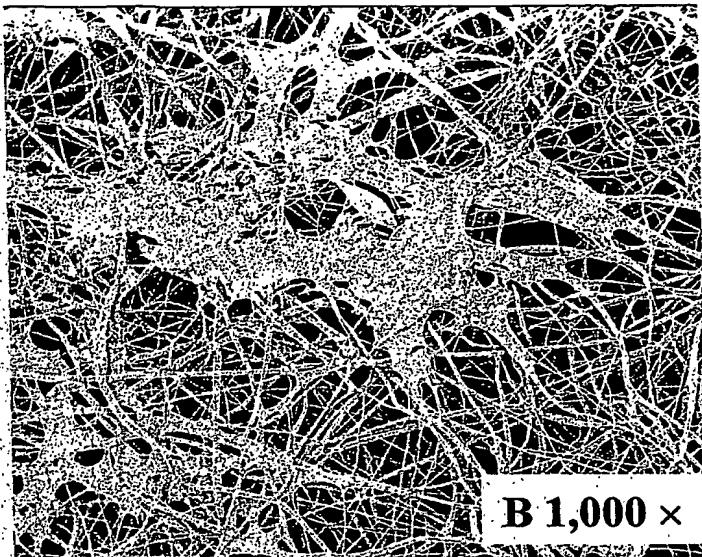


Figure 5. Focused ion beam scan of HBMF cells on PCL-65 after 7 days in cell culture medium.  
Magnification x1000.